Gene Therapy for Duchenne Muscular Dystrophy

Expectations and Challenges

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Duchenne muscular dystrophy is a debilitating X-linked disease with limited treatment options. We examined the possibility of moving forward with gene therapy, an approach that demonstrates promise for treating Duchenne muscular dystrophy. Gene therapy is not limited to replacement of defective genes but also includes strategies using surrogate genes with alternative but effective means of improving cellular function or repairing gene mutations. The first viral-mediated gene transfer for any muscle disease was carried out at Columbus Children’s Research Institute and Ohio State University for limb girdle muscular dystrophy type 2D, and the first viral-mediated trial of gene transfer for Duchenne muscular dystrophy is under way at the same institutions. These studies, consisting of intramuscular injection of virus into a single muscle, are limited in scope and represent phase 1 clinical trials with safety as the primary end point. These initial clinical studies lay the foundation for future studies, providing important information about dosing, immunogenicity, and viral serotype in humans. This article highlights the challenges and potential pitfalls as the field advances this treatment modality to clinical reality.

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Duchenne muscular dystrophy (DMD) is the most common life-threatening childhood form of muscular dystrophy. It is an X-linked recessive disorder with an incidence estimated to be 1 in 3500 live male newborns. Mutations in the dystrophin gene, encoding a large (427-kDa) cytoskeletal protein found in skeletal and cardiac muscle, as well as smooth muscle, brain, and retina, cause DMD. The dystrophin gene is the largest gene identified to date, and because of its size, it is susceptible to a high sporadic mutation rate, ensuring that the disease can never be eliminated. It is estimated that 1 in 10 000 germ cells show de novo mutations, with some estimates as high as twice this number in certain populations. The consequences of the disease are debilitating, usually resulting in death in the early 20s in affected individuals; thus, medicine is challenged to find a treatment. Only corticosteroids have a salutary effect on DMD. Unequivocal proof was established in 1989 in a double-blind, randomized, controlled trial. The wide range of therapeutic prospects includes growth-modulating agents that increase muscle regeneration and delay muscle fibrosis, anti-inflammatory agents, and agents designed to suppress stop codon mutations (aminoglycosides and other agents such as PTC-124 [PTC Therapeutics Inc, South Plainfield, New Jersey]). In this brief review, we examine the possibility of moving forward with gene therapy for muscular dystrophy, addressing topics that will be relevant to musculoskeletal disorders and gene therapy in general.

**CHOICE OF VIRUS FOR GENE THERAPY**

The delivery of “naked” DNA, a rudimentary form of gene therapy, proved to be inefficient and incapable of sustained trans-
gene translation in DMD. In contrast, viruses are ideal vehicles for therapeutic gene transfer, having evolved to infect specific cell populations with high efficiency. The choice of virus is guided by factors including the target cell, immunogenicity, and required duration of transgene expression. Adenovirus was the early favorite for gene transfer to muscle and other tissues including lung (cystic fibrosis) and liver (infectious, neoplastic, and metabolic disorders). Disabling regions for replication (E1 and E2) and regulatory functions (E3 and E4) resulted in recombinant adenoviruses with presumed reduced ability to stimulate immune responses while achieving sustained transgene expression. An extreme modification of adenovirus was the deletion of all viral genes (“gutted”), enabling insertion of expression. An extreme modification of adenovirus was the deletion of all viral genes (“gutted”), enabling insertion of as much as 35 kilobases (kb) of DNA and, thus, more than deletion of all viral genes (“gutted”), enabling insertion of as much as 35 kilobases (kb) of DNA and, thus, more than enough for the entire dystrophin complementary DNA. This approach gained momentum despite several drawbacks including challenges in vector production and concerns about the emergence of replication-competent virus. However, substantial uneasiness about the use of this vector was the outgrowth of the unexpected death of Jesse Gelsinger in 1999 after transfer of the ornithine carbamylase gene by adenovirus. Within hours of intrahepatic administration, Gelsinger experienced severe complications and died 2 days later. The exact cause of his death has never been fully delineated, but the clinical findings overlapped with features of toxic shock syndrome.

After the death of Gelsinger, a member of the parvovirus family, adenov-associated virus (AAV), a small single-stranded DNA virus not associated with human disease, took center stage for viral gene delivery. Adeno-associated virus, a member of the Parvoviridae family, is a Dependovirus, requiring helper functions from other sources, usually viruses, to complete its life cycle. The AAV genome is composed of approximately 4.7 kb containing the replication and encapsidation genes. This viral genome is flanked by two 143-base pair inverted terminal repeats. Most often, adenoviral genes provide the helper functions to activate and express the replication and encapsidation genes required for rescue, replication, and packaging. Herpesvirus can have a similar role in activating the helper genes. A further attraction of the parvovirus family is the recognition of the broad diversity of AAV serotypes and genomic variants. Well over 120 AAV variants have been isolated from various species including non-human primates. As of 2006, 11 AAV serotypes (AAV1-11) had been described as candidates for gene therapy. Advantages of each are under intense investigation to establish specific tropism relevant to the target tissue. The most promising serotypes for transducing skeletal muscle are AAV1, AAV2, AAV6, and AAV8.

Adeno-associated virus vectors have a relatively spotless safety profile. An early concern was site-specific integration on chromosome 19. While this attribute is characteristic of wild-type AAV, it is a rare event with the recombinant form of the virus (rAAV). This was thought to be a potential disadvantage, forcing transgene expression from an episomal location. However, since the observed complications of insertional mutagenesis in patients with severe combined immunodeficiency syndrome, genome integration with the potential to disrupt nearby genes is considered less desirable. Loss of this attribute has not diminished the potential for long duration of transgene expression best explained by the stability of the rAAV vector genome to form stable episomal concatemers.

**IMMUNOGENICITY OF AAV AND POTENTIAL IMPACT ON GENE TRANSFER**

A key issue evolving with the transfer of any gene delivery vehicle is the potential for an immune response. While low immunogenicity was considered a major strength supporting the use of rAAV in clinical trials, a number of observations have recently provided a more realistic view. An obvious barrier to AAV transduction is the presence of circulating neutralizing antibodies that preclude binding of the virion to its cellular receptor. This potential threat can be reduced by prescreening patients for AAV serotype-specific neutralizing antibodies or through the application of therapeutic maneuvers such as plasmapheresis before gene transfer. Alternatively, delivering the virus through an intravenous balloon catheter imparts a protected environment enhanced by the removal of blood from the gene-targeted area, providing a hospitable environment for transduction. Another challenge recently uncovered is the development of a cell-mediated cytotoxic T-cell (CT) response to AAV capsid peptides, limiting transgene expression. In the human factor IX gene therapy trial

**Figure 1.** Structure of dystrophin protein. The N-terminal contains the actin-binding domain. H indicates hinge; CR, cysteine-rich region; CT, C-terminal.
in which rAAV was delivered to liver, only short-term transgene expression was achieved and levels of therapeutic protein declined to baseline levels 10 weeks after vector infusion. This was accompanied by signs of hepatocellular damage (elevation of serum transaminase enzyme values) and a CT response as measured by release of interferon-γ directed toward specific AAV capsid peptides. In this setting, AAV capsid sequences were displayed on the surface of transduced liver cells by major histocompatibility complex class I molecules, leading to invasion by activated CD8-positive CTs. To overcome this response, transient immunosuppression may be required; at least until AAV capsids are completely cleared.

Although data from the hemophilia B trial raise concerns that cannot be ignored, additional findings suggest that T-cell activation requires capsid binding to the heparan sulfate proteoglycan (HSPG) receptor, permitting viroshuttling into a dendritic cell pathway (referred to as cross-presentation). This is typical of the AAV2 (used in the clinical factor IX gene therapy trial) but not for AAV serotypes independent of HSPG binding. Further studies are under way to confirm the privileged immune status of virions deficient in HSPG binding. This is not a trivial issue because gene transfer through a vascular approach with HSPG-deficient AAV serotypes, such as AAV8 (see “Gene Therapy by Vascular Delivery” section), seems promising. If AAV8 falls below the window of detection of the immune system, its potential as a safe and effective gene transfer vehicle is substantially enhanced.

**MODIFYING DYSTROPHIN DNA FOR GENE TRANSFER**

The dystrophin protein has 4 major domains (Figure 1.) as follow: the N-terminal, which binds to cytoskeletal F-actin; a central rod domain composed of 24 spectrinlike repeats; the cysteine-rich domain, which binds to the transmembrane protein α-dystroglycan, linking covalently to β-dystroglycan, which together represent a vital component of the dystrophin glycoprotein complex linking the extracellular matrix to the actin cytoskeleton (Figure 2); and the C-terminal, which is not a prerequisite for maintaining stability of the muscle membrane. The full-length dystrophin complementary DNA is 14 kb, pos-
ing a substantial hurdle to the small packaging capacity of AAV. The concept of small dystrophins that easily fit into rAAV vectors is potentially useful and is based on clinical observations in patients with large gene deletions of the rod domain accompanied by a mild phenotype. The seminal example involves a 61-year-old ambulatory patient with Becker dystrophy with a rod domain deletion (exons 17-48), equivalent to almost half (48%) of the coding region of dystrophin.35 Such findings encouraged a systematic study in dystrophin-deficient (mdx) mice to find the optimal complementary DNA that would both stabilize the sarcolemmal membrane and fit the restrictive capacity of AAV.36 A consensus construct has emerged that permits deletion of the C-terminal, and a rod domain consisting of either 4 (microdystrophin) or 5 or more (minidystrophin) spectrin repeats, combined with removal of the 5’ and 3’ untranslated regions.35-37 An rAAV encoding minidystrophin with these features38 is undergoing clinical testing in a phase 1 gene therapy study by intramuscular injection in the biceps muscle of boys with DMD at Columbus Children’s Hospital and Research Institute, Columbus, Ohio.

Figure 3. Focal vascular delivery of recombinant adeno-associated virus serotype 8–green fluorescent protein (r-AAV8-GFP). Both heads of the gastrocnemius muscle in 6-week-old beagles were treated by focal vascular delivery of rAAV8-GFP through the femoral artery using a fluoroscopy guided catheter. A, At 6 weeks after transfer, more than 80% of the muscle fibers of the gastrocnemius muscle exhibited GFP expression. This section is from the central region of the medial head of the gastrocnemius muscle. B, The hamstring muscle from the same limb that was not targeted by focal vascular delivery shows no GFP expression.

USE OF SURROGATE GENES TO MODIFY THE DYSTROPHIC PHENOTYPE

Evidence from experimental gene replacement to the mdx mouse shows that despite substantial correction of the membrane defect by morphologic criteria (eg, protection against Evans blue dye permeability) and reversal of the phenotype (reduced central nucleation) in animals treated at an early age, there remains a gap in full restoration of contractile properties (protection against eccentric contraction) using minidystrophins and microdystrophins.39,40 This raises concerns that small dystrophin delivery may not restore the phenotype to normal in clinical studies. The extent of correction remains to be established in clinical trials. Other approaches under development may augment the therapeutic effect of the small dystrophins.

The field is beginning to test “booster” genes as a therapeutic strategy. Such approaches include combinations of small dystrophins with overexpression of insulin growth factor-1, in particular, the muscle isoform (mIgf-1),41 or inhibition of the negative muscle growth regulatory fac-
tor myostatin. This approach has recently been illustrated in the mdx mouse, in which coinjection of the rAAV-microdystrophin and rAAV-mlgf-1 vectors resulted in a degree of protection against contraction-induced injury not possible without dual gene therapy.41 Similarly, the inhibition of myostatin results in larger and stronger muscles with conservation of benefits, from mice to humans. In addition to inhibiting myostatin with specific antibodies (as applied in a current clinical trial for muscular dystrophies), follistatin is a potent myostatin antagonist. Adeno-associated virus can deliver the gene for follistatin and, because it is a secretory peptide, very high serum levels can be reached, resulting in muscle hypertrophy at regional and remote sites. When virus is injected into the gastrocnemius and hamstring muscles in animals, substantial increases in muscle mass are noted at the site of injection and at remote sites (Figure 4). We are exploring AAV-mediated gene therapy as a form of combinational treatment of muscular dystrophy that would also be applicable to other forms of muscle disease.

Another novel surrogate gene strategy for muscular dystrophy uses the enzyme CT GalNac transferase, preferably called Galgt2, normally restricted to the neuromuscular junction. At this site, Galgt2 is responsible for glycosylating (adding a sugar moeity, the CT carbohydrate) α-dystroglycan. The CT carbohydrate (a name derived from a common epitope shared with activated CTs) bolsters α-dystroglycan in its linkage to the laminins of the extracellular matrix, further ensuring membrane stability. Overexpression of Galgt2 in transgenic mice25 or by gene transfer using AAV vectors in the ectopic expression of glycosylated proteins (eg, CT carbohydrate–α-dystroglycan), laminins usually confined to the neuromuscular junction (laminin α4 and laminin α5) and utrophin along the entire myofiber. The net result is membrane stability by the substitution of utrophin for dystrophin, creating a utrophin-glycoprotein complex. Studies by colleagues in our Gene Therapy Center42 are laying the groundwork for a clinical trial using the GALGT2 gene.

Another advantage accruing to this approach is that overexpression of this normal gene averts the potential for transgene immunogenicity.

VECTOR PRODUCTION METHODS

Extending gene therapy studies beyond small phase 1 safety trials is an important challenge for vector production. To illustrate this point, if a single human dose is determined to be $10^{13}$ particles for a given application and 1000 patients with DMD are candidates for treatment, $10^{16}$ particles would be required. Based on current vector production standards, each cell produces approximately $10^8$ AAV particles, necessitating $10^{13}$ cells to meet the needs for a clinical trial. In practical terms, this would translate to approximately 100,000 flasks or 1000 L of cells in suspension, emphasizing both the limitations and importance of scalable production methods.

Genetically engineered cell lines can be adapted to overcome this potential barrier to clinical applications.41-48 Three essential components are required for AAV production: the rAAV vector genome, the AAV trans-helper genes (replication and encapsidation), and adenovirus or herpesvirus helper genes delivered in plasmid DNA form or via virus infection. Based on our experience, HeLa cells yield the highest titters of rAAV per cell that we have observed to date.

CONCLUSIONS

Multiple strategies are being developed for gene therapy for muscular dystrophy. Clinically meaningful results are anticipated through optimization of a vascular delivery route using replacement, surrogate, or booster genes. Additional studies are required to further define the immunogenicity of AAV serotypes and transgenes and the potential need for immunosuppression. Successful gene therapy as a treatment for DMD is clearly on the horizon, with the potential to improve the lives of patients with this devastating disease.

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